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Influence of long-term treatment with equine somatotropin (EquiGen®) on gonadal function in stallions with poor semen quality

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Influence of long-term treatment with equine somatotropin (EquiGen®) on gonadal function in stallions with poor semen quality

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1. Summary

The aim of the present study was to investigate the spermatogenic and Leydig cell activity in stallions with impaired semen quality after treatment with equine somatotropin. Experiments were performed using 18 adult clinically healthy stallions with poor semen quality which did not pass breeding soundness evaluation. The animals were randomly divided into a treatment (n=9) and a control (n=9) group. Over a period of 90 days, nine stallions received a daily intramuscular injection of 10 mg recombinant equine somatotropin (EquiGen®, BresaGen Limited, Adelaide, Australia) and 9 control animals were injected with the same amount of physiological saline solution. During and until 2 months after treatment, semen characteristics and daily sperm output as well as plasma testosterone concentrations were determined monthly in all stallions. In addition, testosterone concentration measurement after stimulation with hCG was performed in all animals immediately before and at the end of the treatment period as well as 2 months later. Our results demonstrate that equine somatotropin (EquiGen®) given daily in a dose of 10 mg per animal during 90 days had no significant effect neither on plasma testosterone concentrations and hCG-induced testosterone release nor on semen quality parameters in adult stallions with poor semen characteristics.

2. Zusammenfassung

Einfluss einer Langzeitbehandlung mit equinem Somatotropin (EquiGen®) auf die Hodenfunktion von Hengsten mit schlechter Samenqualität

Ziel der vorliegenden Arbeit war es, die Wirkung von equinem Somatotropin auf die Spermatogenese und Leydig-Zell Aktivität bei Hengsten mir reduzierter Samenqualität abzuklären. Für die Untersuchungen standen 18 adulte, klinisch gesunde Hengste vom Nationalgestüt in Avenches zur Verfügung, welche aufgrund ungenügender Samenqualität als nicht zuchttauglich eingestuft worden waren. Die Tiere wurden zufällig einer Behandlungs- (n=9) und Kontrollgruppe (n=9) zugewiesen. Neun Hengste erhielten täglich, während einer Dauer von 90 Tagen, 10 mg recombinantes equines Somatotropin (EquiGen®, BresaGen Limited, Adelaide, Australia) i.m. injiziert während den 9 Kontrolltieren die entsprechende Menge physiologische Kochsalzlösung verabreicht wurde. Während und bis 2 Monaten nach Ende der Behandlung wurde monatlich bei allen Hengsten die Samenqualität beurteilt sowie die tägliche Spermienproduktion und die Plasmakonzentration von Testosteron bestimmt. Zusätzlich wurde bei allen Tieren unmittelbar vor und nach Ende der Behandlung sowie 2 Monate später eine Stimulation mit hCG vorgenommen. Aufgrund unserer Ergebnisse kann gefolgert werden, dass die tägliche Verabreichung von 10 mg equinem Somatotropin (EquiGen®) während 90 Tagen weder die Samenqualität noch die Plasmakonzentrationen von Testosteron ohne und nach hCG-Stimulation bei Hengsten mit schlechter Samenqualität signifikant beeinflusst hat.

3. Résumé

Influence de la somatotropine équine recombinante (EquiGen®) sur la qualité de la semence et la sécrétion de testostérone chez l'étalon

Chez l'étalon, l'influence de l'hormone de croissance sur la régulation des fonctions reproductives est encore peu connue. Le but de cette étude était d'examiner l'activité des cellules germinales et de Leydig chez l'étalon après un traitement avec de la somatotropine équine. L'expérience a été effectuée sur 18 étalons du Haras National d'Avenches qui n'ont pas passé l'examen de la fonction génitale a cause d'une mauvaise qualité de la semence. Les animaux ont été répartis au hasard en un groupe de traitement (n=9) et un groupe de contrôle (n=9). Pendant une période de 90 jours, neuf étalons ont reçu une injection intramusculaire quotidienne de 10 mg de somatotropine équine recombinante (EquiGen®, BresaGen Limited, Adelaide, Australie) alors que les neuf animaux de contrôle ont reçu, de manière similaire, la même quantité d'une solution physiologique saline. Pendant et jusqu'à 2 mois après le traitement, la qualité de la semence et le nombre total de spermatozoïdes produit par jour, ainsi que la concentration de testostérone plasmatique ont été déterminés chez tous les étalons. De plus, des mesures de la concentration de testostérone après stimulation à l'hCG ont été effectuées chez tous les animaux immédiatement avant et à la fin de la période de traitement, ainsi que 2 mois plus tard. Nos résultats démontrent que l'administration de somatotropine équine recombinante n'a d'effets significatifs ni sur les caractéristiques qualitatives de la semence, ni sur la concentration de testostérone plasmatique et sur la sécrétion de testostérone induite par le test hCG.

4. Introduction

Somatotropin (ST) is a growth hormone (GH) released by the anterior pituitary gland that is essential for normal skeletal development. In recent years, accumulated clinical and laboratory evidence suggests that ST also plays an important role in the reproductive system by influencing gonadal development in young animals and maintaining normal reproductive function in adult animals. GH receptors have not only been detected in the ovary of various species (Mathews et al., 1989; Lobie et al., 1990; Schams and Berisha, 2004) but also in the male reproductive system including Leydig and Sertoli cells, the vas deferens, the prostate gland, the ductus epididymis and the seminal vesicles (Lobie et al., 1990; N'Diaye et al., 2002). Moreover, delayed puberty has been observed in human patients with GH deficiency or GH resistance, and subjects with GH excess as in acromegaly have been known to be infertile (Codner and Cassorla, 2002). Early studies on rats indicated an apparent association of GH-deficiency with both compromised spermatogenesis and impaired sperm motility (Breier et al., 1996; Gravance et al., 1997). Furthermore, treatment of subfertile male GH-deficient rats with recombinant bovine GH, markedly increased sperm motility as well as plasma concentration of insulin-like growth factor-I (IGF-I). IGF-I is synthesized in the liver as well as in the gonads and is directly stimulated by GH (Breier et al., 1996). A recent review (Chandrashekar et al., 2004) reports the presence of IGF-I receptors in the gonads of both male and female, providing further evidence of GH influence on gonadal activity. Finally in clinical trials in subfertile men, sperm motility during GH treatment was significantly increased (Ovesen et al., 1996) whereas in another study GH therapy had no effect in men with idiopathic oligozoospermia (Lee et al., 1995).

Experimental use of GH in order to increase gonadal function has provided promising results in both female and male animals. In the cow, treatment with recombinant bovine somatotropin (bST) has been shown not only to improve oocyte morphology (Roth et al., 2002) but also to enhance fertility in artificially inseminated cows (Santos et al., 2004). Furthermore, the application of bST increased the percentage of transferable embryos in superovulated cows and improved pregnancy rates after embryo transfer (Moreira et al., 2002). In the mature bull, treatment with bST increased the ejaculate volume (Bousquet et al., 2004), sperm motility after

freezing/thawing and the non-return rate after artificial insemination (Sauerwein et al., 2000).

Studies concerning the use of GH in the equine species have shown controversial results. Similarly to GH treatment in the cow, administration of equine somatotropin (eST) to cyclic mares significantly increased the number of small follicles on the ovaries (Cochran et al., 1999). In the stallion, treatment with the somatostatin analogue octreotid resulted in a transient decrease in semen motility and testosterone release after hCG-stimulation (Aurich et al., 2003). Storer et al. (2005) reported that daily administration of recombinant equine GH at 20µg/kg body weight over 21 days increased plasma IGF-I concentrations but had no effect on blood LH, FSH and testosterone concentrations. GH-treated stallions showed higher ejaculate volume, but motility, total sperm and sperm morphology in ejaculates collected 2 months after initiation of the GH treatment were not affected. Nonetheless higher pregnancy rates were observed in mares when bred with stallions showing greater concentrations of IGF-I in seminal plasma, suggesting a positive IGF-I effect on sperm function (Macpherson et al., 2002).

The aim of the present study was to investigate the spermatogenic and Leydig cell activity in adult stallions with poor semen quality after long-term treatment with recombinant equine somatotropin.

5. Material and methods

5.1. Animals

The experiment was carried out using 18 adult clinically healthy warmblood stallions, aged between 7 and 24 years from the National Stud in Avenches (Switzerland). From earlier studies (Janett et al., 2003a; 2003b; 2005) all stallions were known to have poor semen quality and did not pass semen evaluation according to the guidelines of “Clinical Fertility Evaluation of the Stallion” by the Society for Theriogenology (Kenney et al., 1983). The animals were kept in box stalls bedded with straw and were fed hay or haylage, oats, barley, corn and pellets supplemented with minerals. Water was available at libitum. All animals were exercised daily for at least 1 hour.

5.2. Experimental design

Before the onset of the experiment, the stallions were trained to mount the phantom and randomly allocated to a GH treatment (n=9) and a control group (n=9) with similar age distribution. GH treated animals were given 10 mg per day (≈ 20 μg/kg body weight) of recombinant equine somatotropin (EquiGen®, BresaGen Ltd., Adelaide, Australia) dissolved in 2 ml sterile water for injection and the control animals received an equivalent volume of saline solution. In both groups daily injections were applied intramuscularly during a period of 90 days starting in the month of July. During treatment the stallions were regularly examined by a veterinarian and adverse reactions caused by the injections were observed and registered.

5.3. Semen collection and examination

Immediately before, during and until 2 months after the treatment, ejaculates were collected at monthly intervals, for 7 consecutive days each time from July to December. Semen was evaluated on days 5, 6 and 7 of each monthly collection. Ejaculate volume of the gel-free semen was measured with a graduated cylinder. Total and progressive motility, sperm concentration and total sperm were assessed in freshly diluted (INRA 96, IMV, Aîgle, France) semen with a Sperm Analyzer (HTM-IVOS, Version 12, Beverly, MA, USA) using 20 micron standard count analysis chambers (Art. no. SC 20-01-C, Leja, Nieuw-Vennep, The Netherlands) and standardized threshold values for stallion semen. The monthly values for each semen parameter were calculated as mean of the collection days 5-7. The daily sperm output (DSA) was determined as mean of the total sperm from days 5-7 (Love et al., 1991). For morphological examination, five drops of fresh semen collected on day 6 were fixed in 2 mL Hancock solution and smears prepared. At least 200 spermatozoa were subsequently evaluated by phase contrast microscopy (Olympus BX50, UplanF1 100x/1.30) and abnormal spermatozoa classified in major (acrosome defects, nuclear vacuoles, abnormal heads, loose abnormal heads, abnormal midpieces, proximal droplets) and minor (loose normal heads, abnormal tails, distal droplets) defects (Blom, 1973; Jasko et al., 1990).

5.4. Testosterone analysis and hCG-stimulation

Blood samples (EDTA) for testosterone determination were collected monthly on day 7 of semen collection, between 8 and 9 am, by jugular venipuncture. In addition, an hCG-stimulation was performed according to Cox (1989) on all stallions, before and at the end of the treatment period as well as 2 months later. Immediately after drawing the first blood sample, 6000 IU hCG (Chorulon®, Veterinaria AG, Zürich) were injected intravenously and 4 further blood samples were collected at intervals of 15 min. After collection the blood was centrifuged immediately (3000 x *g*, 10 min) and plasma decanted and stored at -80°C until testosterone analysis. Testosterone was determined by electrochemiluminescence immunoassay (Elecsys 2010, Roche Diagnostics, Basel, Switzerland) as described earlier (Wang et al., 2004). The detection limit of the assay was 0.02 ng/mL. All samples were analyzed using a biotinylated monoclonal antibody against testosterone. Cross-reactivity with estrogens and progesterone was < 0.01%, with androstendione 0.91% and with 5-α-dihydrotestosterone 1.89%. For validation of between assay precision a pooled samples were analyzed 6 times a day for a total of 10 days (n = 60) and for within assay precision pooled samples were measured 20 times a day (n = 20). Inter- and intraassay coefficients of variation were 2.2% and 1.4%, respectively.

5.5. Statistical analysis

Data were analyzed using StatView 5.0 software program (SAS Institute, Wangen, Switzerland) and tested for normal distribution. A multivariate analysis of variance (ANOVA) with repeated measures was carried out to assess the effects of group allocation, time of collection and treatment (interaction between group and time) on semen characteristics and on plasma testosterone concentrations. Non-normally distributed values (acrosome defects, testosterone concentration) were logarithmized for this purpose. Group (EquiGen®, control) differences at individual time points were compared by unpaired student's *t*-test in normally (volume, concentration, daily sperm output, total and progressive motility, normal sperm, major defects, nuclear vacuoles, abnormal heads) and by Mann-Whitney test in non-normally distributed variables. The level of statistical significance was assumed as $P < 0.05$.

6. Results

6.1. Plasma testosterone

Results from ANOVA demonstrate that monthly measured plasma testosterone concentrations were significantly influenced by group (P = 0.0207) but neither by time of blood collection (P = 0.1429) nor by treatment (interaction between group and time, P = 0.9568).

Mean monthly testosterone values of the EquiGen® group (range 0.32-0.89 ng/mL) were always higher during the whole experiment compared to control (range 0.26-0.42 ng/mL) stallions (Fig. 1). A significant (P < 0.05) group difference was only present in November.

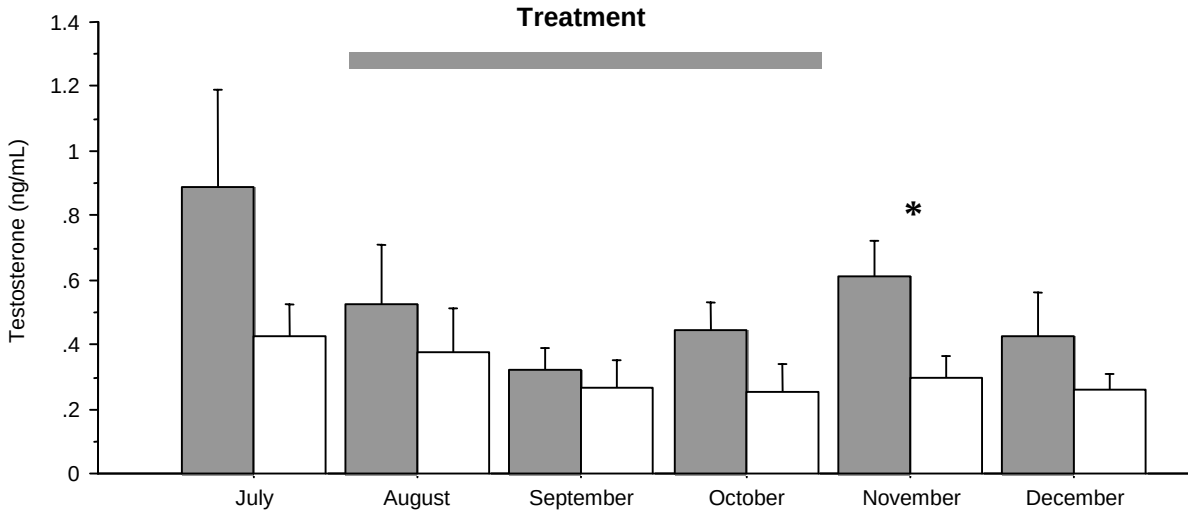

Fig. 1: Mean (± S.E.M.) plasma testosterone concentrations measured monthly in 9 stallions with (■) and 9 stallions without (□) EquiGen®. *Significant difference between groups (P < 0.05, Mann-Whitney test).

Plasma testosterone concentrations in response to hCG-stimulation evaluated by ANOVA showed a highly significant effect of hCG-application (P < 0.0001) with a significant group difference after stimulation (interaction between group and min after hCG, P = 0.0011). There was no significant influence by group (P = 0.1932), single stimulation (P = 0.4507) and treatment (interaction between group, single stimulation and min after hCG, P = 0.9568).

Mean plasma testosterone concentrations in response to hCG stimulation performed before, and at the end of the treatment period and 2 months later are shown in Fig. 2. The stallions showed highly reproducible responses to intravenous injection of hCG, with testosterone concentrations increasing to mean values between 6.42-6.54 ng/mL at 60 min in stallions treated with EquiGen® and between 4.33-4.53 ng/mL in control animals.

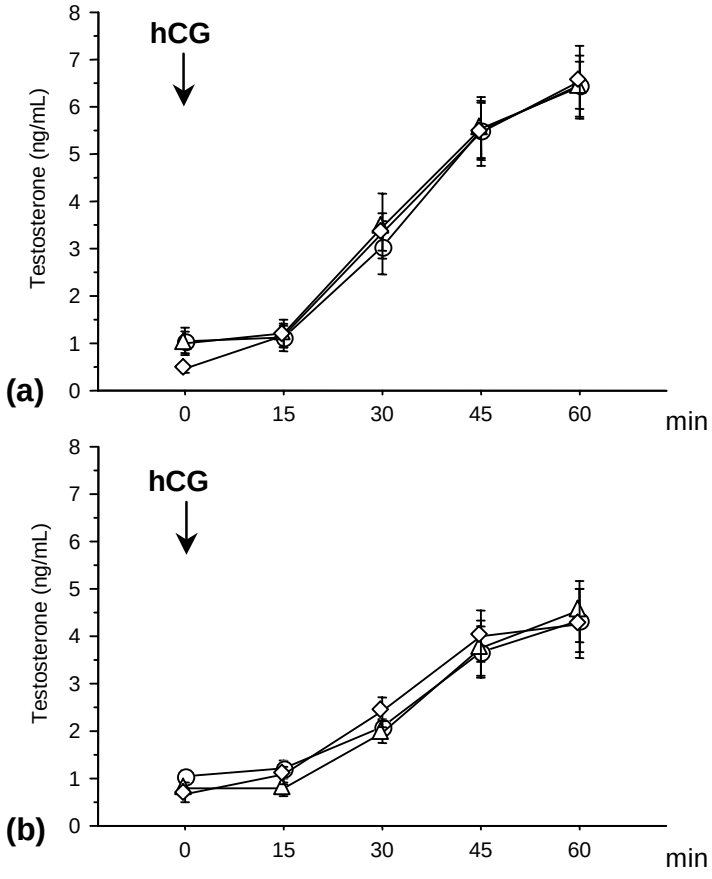

Fig. 2: Mean (± S.E.M.) plasma testosterone concentrations in response to hCG (6000 IU) before (○), at the end of the treatment period (△) and 2 months later (◇) in 9 stallions with (a) and 9 stallions without (b) EquiGen®.

6.2. Semen quality

Data of semen characteristics from ejaculates collected before, during and 2 months after the treatment analyzed by ANOVA are shown in Table 1. None of the parameters measured were significantly ($P > 0.05$) influenced either by group or by treatment (interaction between group and time of semen collection). However, the

time of collection had a significant ($P < 0.05$) effect on volume, concentration, daily sperm output, normal spermatozoa, acrosome defects and abnormal heads in both groups. Changes of mean group values of these parameters during the whole study are shown in Fig. 3 and 4. A significant ($P < 0.05$) group difference in sperm concentration was present in September (Fig. 3b).

Tab. 1: Means (± S.E.M.) of semen characteristics from ejaculates collected from 9 stallions with and 9 stallions without EquiGen® and the effect of group, time of semen collection and interaction between group and time

Parameter	EquiGen® m ± S.E.M.	Control m ± S.E.M.	Group *P*	Time *P*	Interaction *P*
Volume (mL)	28.9 ± 2.2	24.5 ±12.9	0.4455	0.0115*	0.1909
Concentration (x 10^6/mL)	241.8 ±15.4	175.4 ±13.4	0.0907	0.0013*	0.4258
Daily sperm output (x 10^9)	5.6 ± 0.3	3.8 ± 0.4	0.0922	0.0165*	0.8587
Total motility (%)	75.1 ± 1.6	75.1 ± 1.5	0.9979	0.3323	0.9505
Progressive motility (%)	50.5 ± 2.0	52.2 ± 2.1	0.7976	0.3103	0.9792
Normal spermatozoa (%)	18.6 ± 2.4	22.2 ± 2.4	0.6687	0.0338*	0.2318
Major sperm defects (%)	74.8 ± 2.7	70.5 ± 2.5	0.6375	0.0686	0.1007
Acrosome defects (%)	16.0 ± 2.1	7.6 ± 1.1	0.1481	0.0093*	0.6691
Nuclear vacuoles (%)	15.0 ± 1.6	17.8 ± 2.6	0.7096	0.0533	0.4929
Abnormal heads (%)	33.7 ± 2.6	26.1 ± 2.1	0.3307	0.0308*	0.3975

**Significant (P < 0.05)*

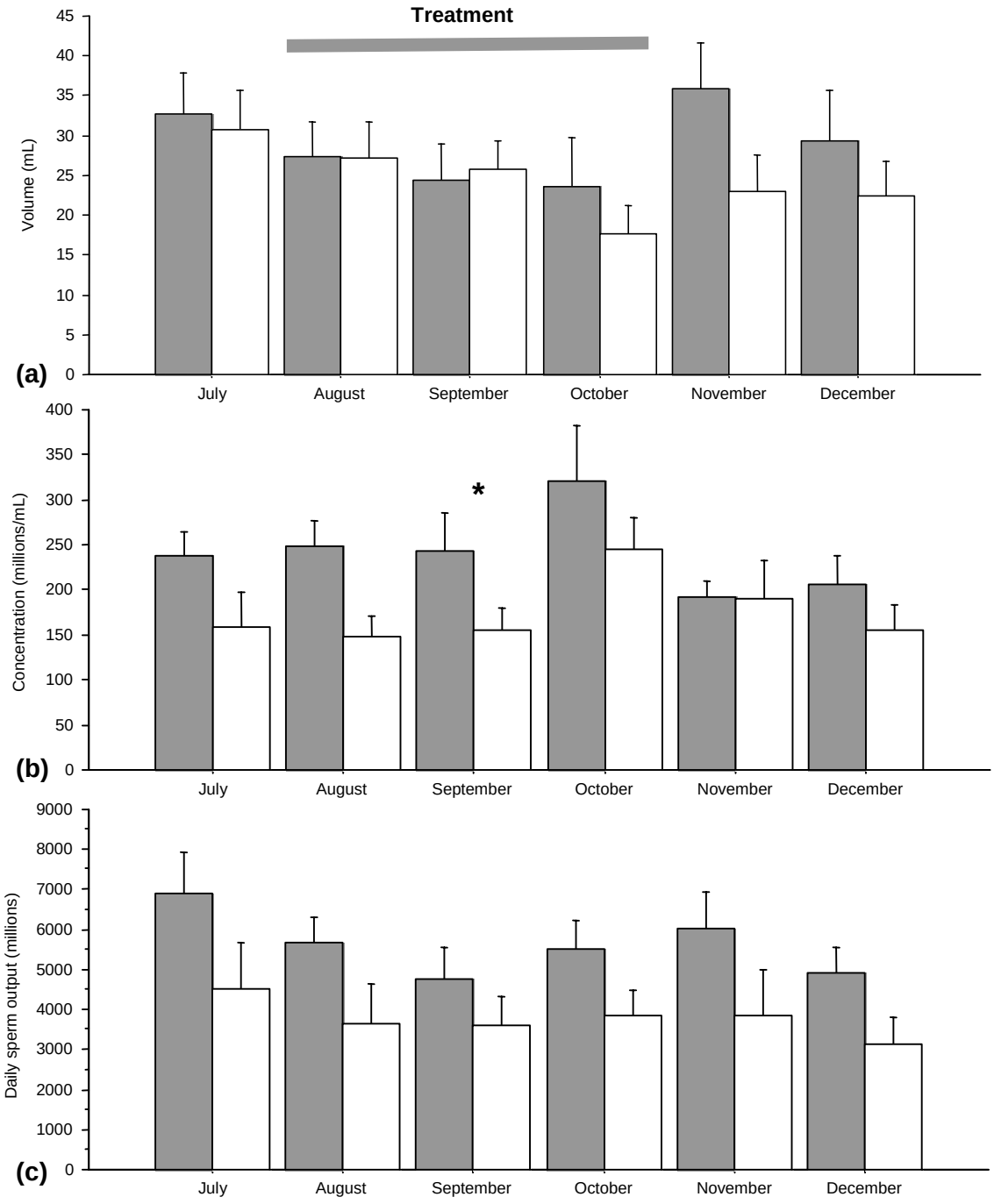

Fig. 3: Mean (± S.E.M.) volume (a), sperm concentration (b) and daily sperm output (c) in ejaculates collected monthly from 9 stallions with (■) and 9 stallions without (□) EquiGen® *Significant difference between groups ($P < 0.05$, unpaired student's t-test) difference between groups.

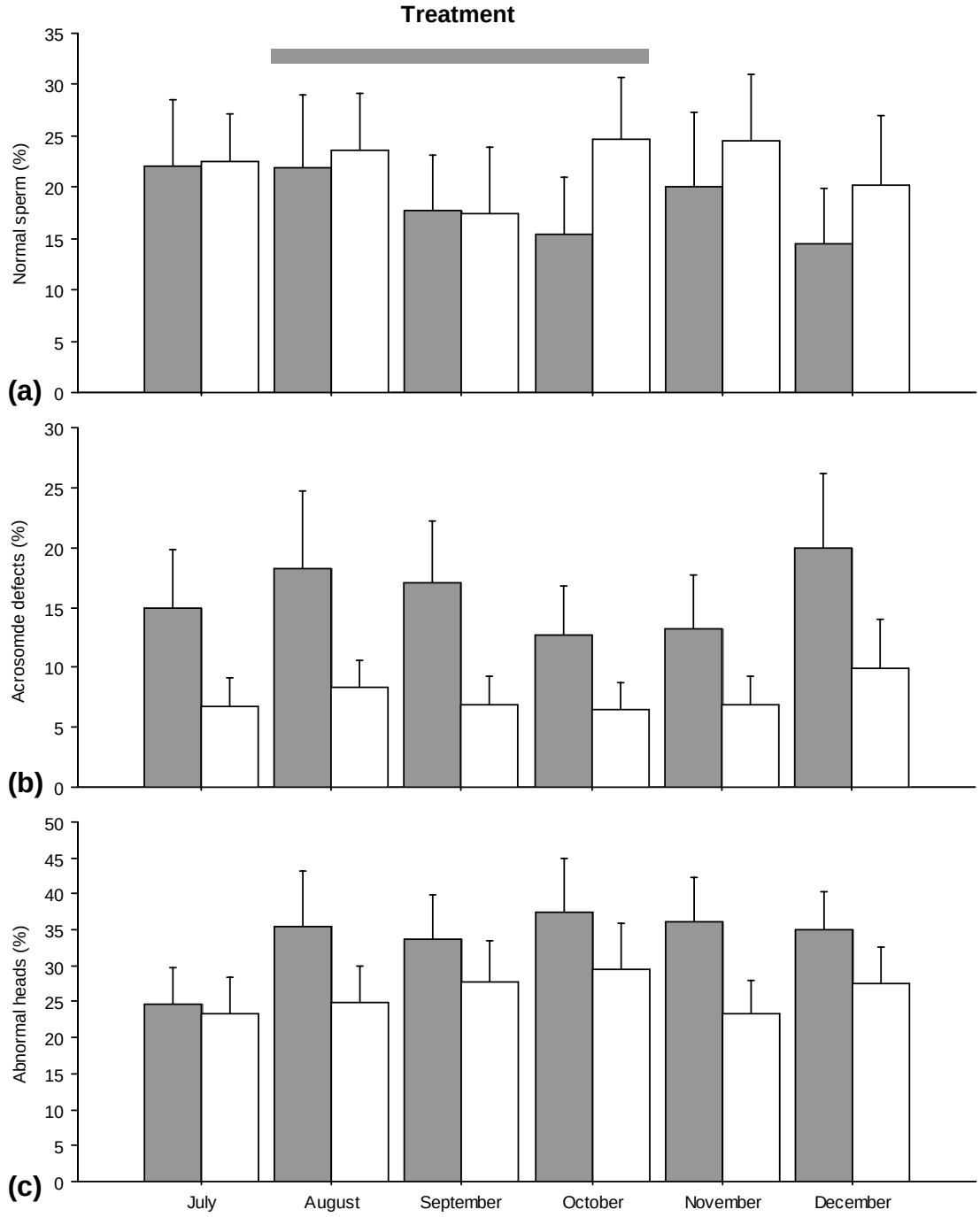

Fig. 4: Mean (± S.E.M.) percentage of normal sperm **(a)**, acrosome defects **(b)** and abnormal heads **(c)** in ejaculates collected monthly from 9 stallions with (■) and 9 stallions without (□) EquiGen®.

6.3. Adverse effects

Adverse effects were observed in 6 of 9 stallions during the first month of EquiGen® treatment. The most prominent reactions were swelling at the injection site, apathy and pyrexia. Animals with elevated body temperature above 38.5°C were treated with vedaprofen (Quadrisol®, Intervet International BV, The Netherlands) a non steroidal anti-inflammatory drug known to have no influence on semen quality (Janett et al., 2005).

7. Discussion

Results of our investigation demonstrate that 10 mg recombinant equine somatotropin (EquiGen®) administered once daily during 90 days did not significantly influence plasma testosterone concentrations or semen quality. A seasonal pattern of testosterone secretion, with lowest values during winter, as reported by others (Byers et al., 1983; Cox et al., 1988; Clay and Clay, 1992; Inoue et al., 1992; Aurich et al., 2003) was not seen in our study. A seasonal effect of testosterone was also absent after hCG-stimulation, performed in July, October and December. These findings may be explained by the short experimental period of only 6 months lasting from midsummer (July) until early winter (December) and by the low sampling frequency for basal and stimulated testosterone concentrations. Based on episodic testosterone secretion in the stallion (Byers et al., 1993; Clay and Clay, 1992) a seasonal pattern can not be identified by obtaining only single monthly values. Irrespective of treatment, the hCG-induced testosterone release after each stimulation differed not significantly. From these results it can be concluded that the daily injections of EquiGen® did not improve Leydig cell function either by increasing the cell number or their sensitivity for LH. This observation corresponds with the results of Storer et al. (2005) who demonstrated that daily intramuscular injections of 20 µg/kg body weight (an equivalent dose as used in our experiment) EquiGen® for 21 days in 5 stallions increased IGF-I concentrations but did not influence pituitary gonadotrope function or testosterone secretion. In contrast to the horse, the use of GH in rodents has been shown to stimulate testosterone secretion (Horikawa et al., 1989; Ohyama et al., 1995].

In order to compare daily sperm output and semen quality over time, ejaculates were collected using a standardized protocol with depletion of extragonadal sperm

reserves during 4 consecutive days followed by 3 further daily collections for determination of semen characteristics (Love et al., 1991). During the entire experimental period of 6 months, some of the semen characteristics (volume, concentration, daily sperm output, normal sperm, acrosome defects, abnormal heads) showed significant variations in both groups over time. These differences in semen quality may be explained by a seasonal effect as shown in previous studies (Janett et al., 2003a; 2003b). An effect on semen quality parameters attributable to EquiGen®, however, was not obvious. Storer et al. (2005) also looked at testicular function in the stallion, and reported an increased ejaculate volume as the only measurable effect after EquiGen® application, with semen being collected every other day for 14 days beginning 2 months after initiation of the treatment. This study, however, was carried out during the non breeding season and EquiGen® was given during 21 days only which makes a comparison with our data difficult. The lack of clear somatotropic action on gonadal function in the stallion is difficult to interpret and is supported by in vitro experiments showing that equine GH and humane recombinant IGF-I had no steroidogenic effect on Leydig cells (Hess and Roser, 2005).

In the aged bull, subcutaneous treatment with 640mg recombinant bovine GH every two weeks over 14 weeks was shown to have no effect on blood testosterone concentration. It did, however, increase sperm motility after freezing-thawing and non-return rate after artificial insemination (Sauerwein et al., 2000). The effect on sperm motility after freezing may be more attributable to GH induced changes in the spermatozoal environment, as a direct influence of GH on spermatogenesis could not be show in the bull (Santos et al., 1999). This is in agreement with earlier reports where GH supplementation to prepubertal bulls (MacDonald and Deaver, 1993;Hagen et al., 1991) and boars (Swanlund et al., 1995) did not improve testicular development. In the young boar, however, daily treatment with porcine GH from 8 through 40 days of age promoted tubular and Sertoli cell maturation without any detectable change in plasma testosterone concentration (Swanlund et al., 1995). From reports in humans (Laron, 1984) and animal models (McDonald and Deaver, 1993; Ohyama et al., 1995; Swanlund et al., 1995; Breier et al., 1996; Gravance et al., 1997) we can conclude that a beneficial effect of somatotropin on male

reproductive performance is likely to be confined to prepubertal individuals or growth hormone deficient patients.

Adverse effects with reactions including swelling and pain at the injection site as well as pyrexia were observed in 6 of 9 stallions given EquiGen® during the first month of treatment. To minimize side effects we recommend halving the dose (5mg/day) during the first week of treatment and to rotate the daily intramuscular injections between at least 4 different injection sites.

In summary it seems that long-term treatment with EquiGen® did not influence plasma testosterone concentrations nor semen quality in clinically healthy adult stallions with poor semen quality.

8. Acknowledgement

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